RESEARCH ARTICLE

Antiviral and Immunomodulatory Effects of Dipotassium Glycyrrhizinate in Chicks Artificially Infected with Infectious Bursal Disease Virus

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A B S T R A C T

Glycyrrhizinate dipotassium (DG) is a widely used anti-inflammatory, anti-microbial and anti-viral agent isolated from the licorice root. This study aims to investigate the potential antiviral activity of DG and its underlying mechanisms of protection against infectious bursal disease virus (IBDV) *in vivo*. The international guiding principles for Biomedical Research Involving Animals were followed during animal experiment. Measured the indices of immune organs including spleen, thymus and bursa of Fabricius, level of anti-IBDV antibody (Ab), IFN-γ and IFN-β in serum through ELISA, lymphocyte proliferation and the ratio of CD4$^+$/CD8$^+$ in blood using MTT and flow cytometry respectively and the expression of IBDV VP1 and TLR3 host gene in bursa of Fabricius were also evaluated by qRT-PCR. The DG treatment groups showed increased immunological index of spleen, thymus and bursa of Fabricius, a much higher ratio of CD4$^+$/CD8$^+$, the levels of anti-IBDV Ab, IFN-γ and IFN-β in serum were up-regulated varyingly, inhibit the VP1 expression and also up-regulated the TLR3 expression in bursa of Fabricius. These findings suggested that DG inhibited IBDV infection *in vivo* through multiple pathways and DG is a promising drug candidate for further development.

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INTRODUCTION

Infectious bursal disease virus (IBDV) causes a highly contagious disease in chicks via functional incapacitation of the bursa of Fabricius and inflammation, leading serious economic losses to poultry industry (Müller *et al.*, 2003; Smith *et al.*, 2015). IBDV including two segments, both encodes Viral proteins (VPS): segment A encodes VP2, VP3 and VP4 while segment B encodes VP1 and VP5. Previous studies have demonstrated that VP1 of IBDV represents the RNA-dependent RNA polymerase and it is an essential protein for the replication of IBDV (Tacken *et al.*, 2000; von *et al.*, 2004; Wang *et al.*, 2013). Further, Gao *et al.* (2017) demonstrated that viral polymerase activity was inhibited through binding to VP1. In the host immune system, Toll-like receptors (TLRs) are important for the detection of sensors of antiviral innate immunity (Li *et al.*, 2005).

Among TLRs, TLR3 acts as a mediator of dsRNA signaling, can activate several important antiviral pathways by stimulating the transcription factors, such as IRF3 (interferon regulatory factor 3) and NF-κB in IBDV infection models (Raj *et al.*, 2011; Rauf *et al.*, 2011a; Smith *et al.*, 2015).

Vaccination against infectious bursal disease is necessary to protect chicks against infection during their first week after hatching. However, there are several drawbacks during the vaccination process. Such as, hot vaccines may cause lesions in the bursal lymphoid follicles and induce immunosuppression as well as carry the risk of reversion to virulence (Berg, 2000; Müller *et al.*, 2003). Relatively, natural compounds possessed multi-pharmacological properties including antiviral (Ou *et al.*, 2012; Ganguly *et al.*, 2018), anti-inflammatory (Ye *et al.*, 2016) and immune-regulation effects (Maroufyan *et al.*, 2013) etc. So far, several natural compounds have been screened in our previous researches and their antiviral effects have been confirmed (Cheng *et al.*, 2013;

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Sun *et al*., 2013; Sun *et al*., 2014). Among them, Dipotassium Glycyrrhizinate (DG), a derivative of glycyrrhetinic acid, comprises the antiviral activity against IBDV (Sun *et al*., 2013) and porcine reproductive and respiratory syndrome virus (PRRSV) *in vitro* (Wang *et al*., 2013). In this study, we further investigated the potential antiviral activity of DG and its underlying mechanisms of protection against IBDV *in vivo*.

MATERIALS AND METHODS

Virus and drugs: IBDV-BC6/85 strain was provided by China Institute of Veterinary Drugs Control (Beijing, China). In this study, the virus ID_{50} was $10^{-5.2}$/0.1 mL. DG was purchased from Rui Hong biological technology (Xi'an, China) and its purity was 90.9% according to HPLC analysis (Data not shown). Ribavirin (RV) was obtained from Yong Heng animal husbandry pharmaceutical (Shenzhen, China) and its purity was greater than 98%.

Experimental design: The 24 days-old Hy-line variety brown chicks, negative for antibodies against IBDV, were randomly divided into seven groups (25 each) as shown in Table 1. Except for normal control group (challenged with PBS only), all were intranasally inoculated with 0.2 mL $100ID_{50}$ IBDV at 28 days old. Ribavirin (26 mg/kg) or DG (80, 40 and 20 mg/kg) were given in the drinking water respectively during 31 to 36 days old and DG preventive group, DG (40 mg/kg) were also given in drinking water from 25 to 30 days old. At 27, 30, 37 and 44 days old, three chicks per each group were randomly selected, weighed and indices of spleen, thymus and bursa of Fabricius were measured after postmortem examination according to the formula: the immune organ index (mg/g) = immune organ weight / body weight. The international guiding principles for Biomedical Research Involving Animals were followed during animal experiment.

T lymphocyte proliferation assay: At 27, 30, 37 and 44 days, blood samples were taken and lymphocytes were obtained according to the previous description (Zhang *et al*., 2013) from the remaining chicks of each group. 1×10^6 cells in 100 μL medium per well were seeded into 96-well plates in triplicate, followed by the addition of 10 mg/L ConA (100 μL) per well and the addition of 100 μL cell maintenance medium to the control group. Post 48 h incubation, MTT assay was used to detect the T lymphocyte proliferation. The stimulation index (SI) of lymphocyte proliferation was calculated according to the following formula: SI = treated group OD_{490} / cell control group OD_{490}.

Determination of CD4$^+$/CD8$^+$: CD4$^+$/CD8$^+$ in peripheral blood were measured by flow cytometry. Cell samples

were prepared according to the lymphocyte proliferation assay. Lymphocytes were stained with both FITC-conjugated mouse anti-chicken CD4 antibodies (C1706-PM70F, Southern Biotech, USA) and PE-conjugated mouse anti-chicken CD8 (G149-VJ39R, Southern Biotech, USA). The cell samples were analyzed on a FACS Calibur flow cytometer (BD Biosciences, USA). 10000 events for single sample were analyzed to evaluate FITC and PE positive signals. Results for CD4$^+$ or CD8$^+$ were expressed as percentages of events in the forward scatter characteristics /side scatter characteristics of gated lymphocyte.

Measurements of anti-IBDV antibody, IFN-γ and IFN-β level in serum: Serum level of anti-IBDV antibody (Ab), IFN-γ and IFN-β on 27, 30, 37 and 44 days were measured, respectively using ELISA kits according to the manufacturer's instructions (RapidBio Lab, USA). The OD value was read at 450 nm with an Absorbance Microplate Reader. The concentrations of anti-IBDV Ab, IFN-γ and IFN-β were determined by extrapolation from their respective standard curves according to the manufacturer's protocol.

qRT-PCR analysis of IBDV VP1 and TLR3 host gene in bursa of Fabricius: Total RNA was extracted from bursa of Fabricius of each group on 30, 37 and 44 days using TRIzol regent (Invitrogen, USA) and transcribed reversely to cDNA with PrimeScriptTM RT reagent Kit with gDNA Eraser (Takara, China). Primers are shown in Table 2. The relative quantities of VP1 and TLR3 gene were calculated using $2^{-\triangle\triangle Ct}$ method.

Statistical analysis: Data were analyzed using one-way ANOVA implemented in GraphPad Prism 5 software and expressed as mean±SEM, P<0.05 was considered statistically significant.

RESULTS

DG up-regulated the serum anti-IBDV Ab level: The effects of DG on IBDV-Ab expression in IBDV-challenged chicks were evaluated. As shown in Fig.1, comparing with the normal control group, the titer of the anti-IBDV antibody in the IBDV control group was significantly increased at 30 and 37 days old (P<0.05). At 37 days old, the titer of the anti-IBDV antibody increased in all DG treatment groups comparing with the IBDV control (P<0.05). Moreover, the titers of anti-IBDV antibody in DG treatment groups were much higher than that in Ribavirin treatment groups. At 44 days old, comparing with IBDV control group, DG different dose treatment groups had a significant increased (P<0.05). These results suggested that DG could enhance the titer of the anti-IBDV antibody.

Table 1: Experiment design and treatment

Groups	Chick age of IBDV infection (days)	Dosages (mg/(kg•d))	The route of administration	Chick age (days) at the time of DG
Normal control group*	-	-	-	-
IBDV control group	28	-	-	-
Ribavirin treatment group	28	26	oral	31 -36
DG preventive group	28	40	oral	25 -30
DG high-dose treatment group	28	80	oral	31 -36
DG moderate-dose treatment group	28	40	oral	31 -36
DG low-dose treatment group	28	20	oral	31 -36

Note：Normal control group represent chicks that were challenged with PBS instead of IBDV and with no treatment.
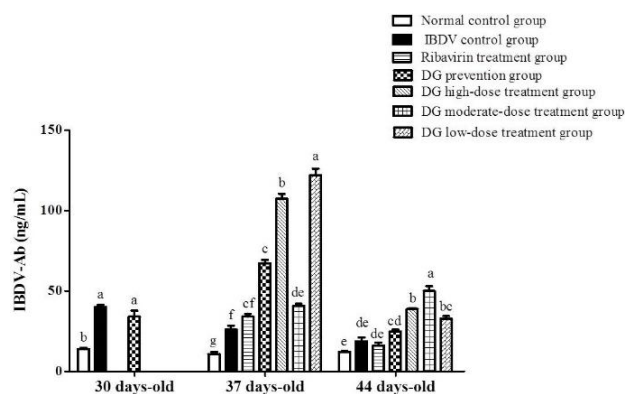
Fig. 1: Impact of DG on anti-IBDV antibody in serum. Following IBDV inoculation and DG treatment, the serum anti-IBDV antibody level was monitored by ELISA at 30, 37 and 44 days old. DG could increase the production of anti-IBDV antibodies. Data were expressed as mean ± SEM of 3 chicks. Data with different letters (a-g) indicate significant differences between groups (P<0.05).

Table 2: Primer sequences for qRT-PCR

Gene name	Primer sequences	Accession No.
VP1	Forward: AGCAGCGTTCGGCATAAA Reverse: GTCGGTCTCATACTCCTCATTCT	DQ 906922
TLR3	Forward: GGCTTGGACATTTCCCATAA Reverse: TTTGCACAATCCTGGTTTCA	NM 001011691
GADPH	Forward: AGGGTGGTGCTAAGCGTGTTA Reverse: TCTCATGGTTGACACCCATCA	NM 204305

Effects of DG on the indices of immune organs: In order to gauge immunological responses at organs level, the indices of the immune organs were analyzed following the IBDV inoculation and DG treatment. As shown in Fig.2A, the indices of the thymus and bursa of Fabricius for the 27 days old chicks in DG preventive group were significantly increased comparing with those in the normal control group (P<0.05). At 30 days old chicks in IBDV group (Fig.2B), the index of the bursa of Fabricius was much higher than those of the other groups (P<0.05). At 37 days old (Fig. 2C), comparing with normal control group, the indices of thymus and bursa of Fabricius in IBDV group decreased significant (P<0.05) and the DG high-dose treatment group improved spleen index (P<0.05); comparing with IBDV control, only Ribavirin treatment group enhanced bursa of Fabricius index (P<0.05) and thymus index (P>0.05). Moreover, the other treatment groups have no significant influence on the indices of the immune organs (P>0.05). At 44 days old (Fig. 2D), the indices of the spleen and bursa of Fabricius in the IBDV group were significantly decreased comparing with the normal control group (P<0.05). When compared with IBDV control group, the index of the spleen in the DG preventive group and the DG low-dose treatment group were significantly improved (P<0.05), and DG low-dose treatment group also increased the index of thymus (P<0.05); the rest of other treatment groups have a little enhanced but no statistical difference on immune organ indices (P>0.05).

Impact of DG on T lymphocyte proliferation and the ratio of CD4⁺/CD8⁺ in peripheral blood: T cell mediated immunity plays an essential role in the clearance of IBDV and the stimulating index of peripheral T lymphocyte proliferation was measured as previously described at 27, 30, 37 and 44 days old. The results showed that IBDV infection had no significant impact on T lymphocyte proliferation (P>0.05). When compared with IBDV control group, after treatment with DG or Ribavirin, there also had no significant difference in T lymphocyte proliferation but the Ribavirin and 80 mg/kg DG had a certain T lymphocyte proliferation at 37 and 44 days old (P>0.05) (Fig. 3C and 3D).

The ratio of CD4⁺/CD8⁺ could be used as a cellular immune index to evaluate immune function. As shown in Fig. 3A and 3B, the ratio of CD4⁺/CD8⁺ for 27 and 30 days old chicks in DG preventive group was significantly enhanced when compared with normal control or IBDV control group (P<0.05). Compared with IBDV control, the ratio of CD4⁺/CD8⁺ increased in all groups treated by DG and was the highest at 37 days old chicks in DG preventive group (P<0.05) (Fig. 3C). At 44 days old (Fig. 3D), there was no remarkable effect on the ratio of CD4⁺/CD8⁺ between all treatment groups with DG and IBDV control.

Effect of DG on the VP1 and TLR3 expression in Bursa of Fabricius: VP1 is an essential structural protein for the replication of IBDV, the relative quantities of VP1 were monitored and calculated using qRT-PCR by $2^{-\triangle\triangle Ct}$ method. As shown in Fig. 4A, comparing with IBDV control group, the expression of VP1 was significantly decreased in all treatment groups (P<0.05), except for DG low-dose treatment group at 37 days old. Such inhibition of the VP1 expression by DG was does dependent at 37 days old chicks.

As shown in Fig. 4B, the TLR3 expression in IBDV control was much higher than normal control at 37 (P>0.05) and 44 (P<0.05) days old. Compared with IBDV control, the expression of TLR3 was significantly inreased in Ribavirin, DG preventive and DG moderate-dose treatment groups at 37 days old (P<0.05) and there has no significant change at 44 days old (P>0.05). These results showed that DG could promote the possible host-directed defense responses against the virus.

DG up-regulated the serum IFN-γ and IFN-β levels: As shown in Fig. 5A and 5B, the expression of IFN-β for 27 and 30 days old chicks in DG preventive group had no significant changes when compared with normal control or IBDV control (P>0.05). At 37 days old (Fig. 5C), comparing with the IBDV control, DG preventive and Ribavirin treatment group increased the IFN-β levels (P>0.05). At 44 days old (Fig. 5D), only DG low-dose treatment group enhanced the IFN-β level when compared with IBDV control group (P<0.05); the other treatment groups have no statistical difference on immune organ indices (P>0.05).

Comparing with the normal control group, the IFN-γ was statistically higher (P<0.05) in the DG preventive group at 27 days old (Fig. 5A) and the DG high-dose, Ribavirin group at 37 days old (Fig. 5C). At 44 days old (Fig. 5D), IBDV enhanced the expression of IFN-γ when compared with normal control group (P<0.05); comparing with IBDV control, DG preventive and DG low-dose treatment group significant decreased the IFN-γ expression (P<0.05).
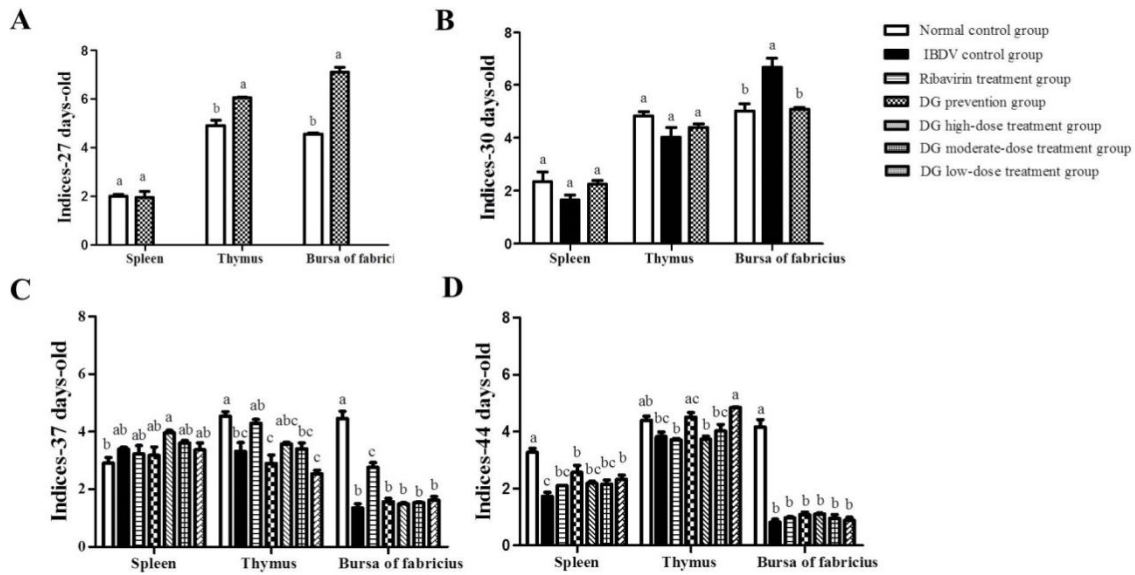
Fig. 2: Effects of DG on the indices of the spleen, thymus and bursa of Fabricius at 27 (A), 30 (B), 37 (C) and 44 (D) days old. The indices of the spleen, thymus and bursa of Fabricius were calculated according to the weight of the spleen, thymus or bursa of Fabricius to the body weight (mg/g). Data were expressed as mean±SEM of 3 chicks. Data with different letters (a, b, c) indicate significant differences between groups ($P<0.05$).
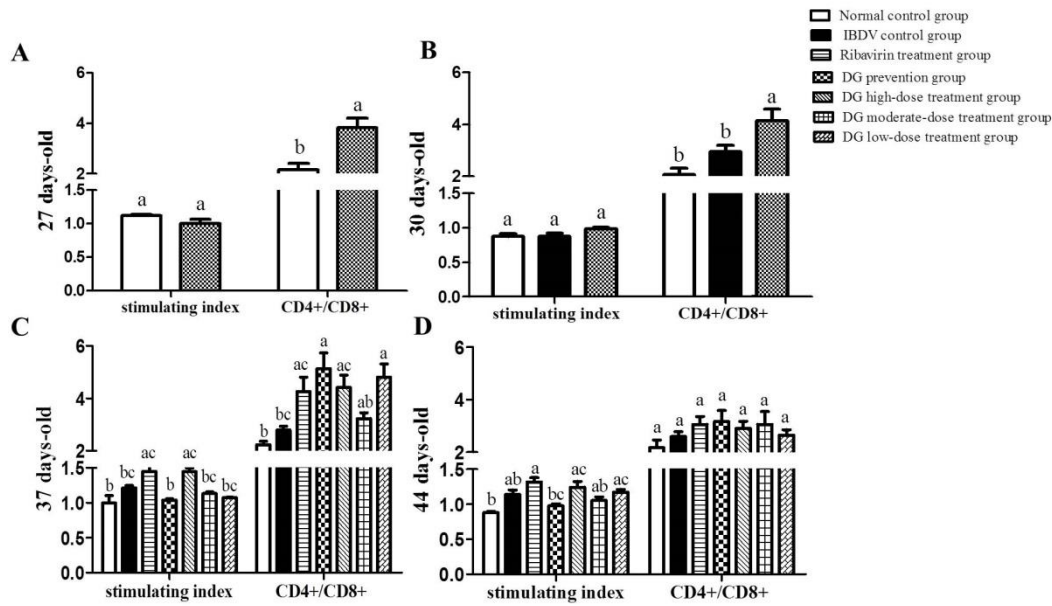

Fig. 3: Lymphocyte proliferation and the ratios of CD4^{+}/CD8^{+} in peripheral blood. The lymphocytes in peripheral blood were collected at 27 (A), 30 (B), 37(C) and 44 (D) days old. $P<0.05$ was considered statistically significant. Data were expressed as mean ± SEM of 3 chicks. Data with different letters (a, b, c) indicate significant differences between groups ($P<0.05$).
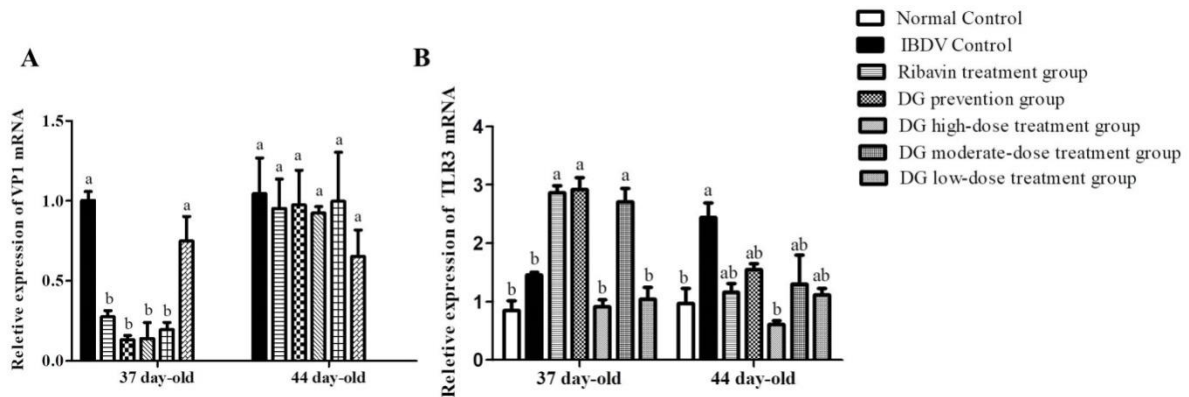

Fig. 4: qRT-PCR analysis IBDV VP1 and host gene TLR3 in bursa of Fabricius. The data were expressed as the average ratio of VP1 (or TLR3)/GADPH. $P<0.05$ was considered statistically significant. Data were expressed as mean ± SEM of 3 chicks. Data with different letters (a, b) indicate significant differences between groups ($P<0.05$).
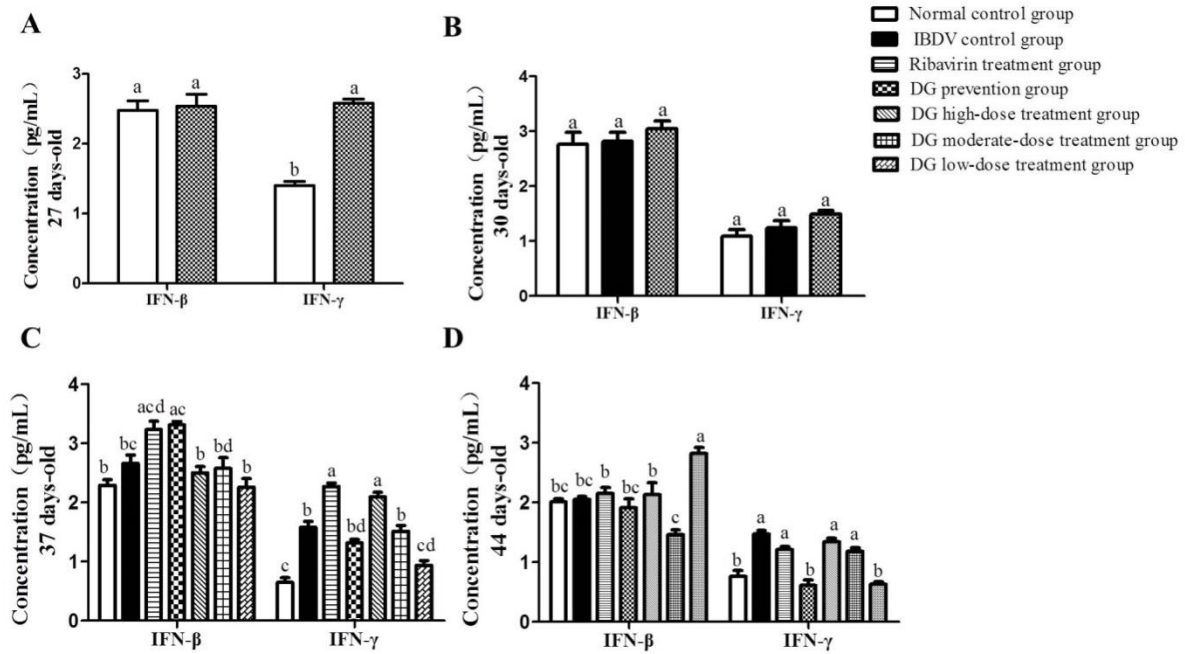
Fig. 5: The regulation of DG on serum IFN-γ and IFN-β level. Serum in different treatment groups were isolated at 27 (A), 30 (B), 37 (C) and 44 (D) days old. Note: Data were expressed as mean ± SEM of 3 chicks. Data with different letters (a, b, c, d) indicate significant differences between groups (P<0.05).

DISCUSSION

Some derivatives of glycyrrihizin have been demonstrated to have antiviral activities. Such as glycyrrhizin has been identified as a novel anti-hepatitis C virus agent and it also inhibits human parainfluenza virus type 2 replication by the inhibition of genome RNA, mRNA and protein syntheses (Matsumoto *et al.*, 2013; Sakai-Sugino *et al.*, 2017). Diammonium glycyrrhizinate could inhibit pseudorabies virus (PrV) replication through reducing cell apoptosis induced by PrV infection (Sui *et al.*, 2010). Our previous results have demonstrated that DG could inhibit IBDV infection *in vitro* (Sun *et al.*, 2013). In this study, we further explore the antiviral activities of DG *in vivo*.

The level of anti-IBDV antibody can be used to evaluate the protection against IBDV infection *in vivo*. In this study, the titer of anti-IBDV antibody in DG treatment groups significantly increased compared with that of Ribavirin treatment groups (P<0.05). Moreover, DG could increase the index of bursa of Fabricius. As bursa of Fabricius plays a vital role in the production of antibodies in chicks, our results suggest that DG promote the development of bursa of Fabricius to produce more antibodies against IBDV infection.

Both innate and acquired immune responses in chicks could be compromised by the immunosuppression induced by virus infection (Wei *et al.*, 2012). Spleen, thymus and bursa of Fabricius are important immune organs of chicks and their indices could reflect immune functions. Wei *et al.* (2012) have demonstrated that increase of spleen and thymus indices by carboxymethylpachymaran administration correlates well with the immune response against the immunosuppression induced by PCV2 in mice. In this study, IBDV infection suppressed the development of the immune organs and DG could improve the development of the immune

organs. T cell mediated immunity plays an essential role in the clearance of IBDV (Williams and Davison, 2005). Rauf *et al.* (2011b) showed that CD4^{+} and CD8^{+} T cells enter the infected bursa, and that cytotoxic T cells play a role in clearing infected cells through the perforin-granzyme pathway. Present study found that DG high-dose treatment group slightly increased stimulating index and DG treatment groups, especially DG preventive treatment group, induced a significantly higher ratio of CD4^{+}/CD8^{+}, suggesting DG had a positive effect on enhancing cellular immunity. CD4^{+} T cells play a major role in mediating immune response through the secretion of specific cytokines. After activation, CD4^{+} T cells differentiate into distinct effector subtypes, termed T-helper 1, T-helper 2, T-helper 17, regulatory T cell, follicular helper T cell, and T-helper 9. IFN-γ and IFN-β secreted by Th1 cells are involved in cell-mediated immune response (Zhang *et al.*, 2014). Previous researches have also demonstrated that IFN-γ and IFN-β were involved in virus replication and antiviral response (Jain *et al.*, 2013; Dar *et al.*, 2014), present study showed that the expression of IFN-γ was increased in IBDV-infected chicks, and DG could inhibited the expression of IFN-γ.

The RNA dependent RNA polymerase of virus is responsible for virus replication. Many researchers have focused on this target to develop new antiviral drugs (Groskreutz *et al.*, 2006; Kang and Lieberman, 2011; Gao *et al.*, 2013; Liu *et al.*, 2015). In this study, we evaluated the effect of DG on IBDV replication by evaluating the expression of VP1 and our results showed that all DG treatment groups significantly inhibited VP1 expression in a dose-dependent manner. TLR3 has been reported to play a protective role in viral infection and induce Type I IFN to reduce IBDV titer (Groskreutz *et al.*, 2006; Wong *et al.*, 2007). Present study showed that DG preventive group up-regulated TLR3 expression, indicating that DG

could inhibit IBDV VP1 expression by enhancing TLR3 expression which is crucial during innate immune response.

Conclusions: Our findings demonstrated that DG inhibits IBDV infection in chicks' models via activating multiple targets. We recommend that DG should be further investigated to explore its antiviral spectrum and the antiviral mechanisms in order to develop better and more potent antiviral drugs with wider applications.

Authors contribution: NS, NX, YS and HL designed all the experiments. NS, PS and NX performed the cell culture, qPCR assay and Western blot assay. KF and WY do the flow cytometry. NS wrote the manuscript and AK revised this manuscript.

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